

## FLUORESCENT PROTEINS

### Field of the Invention

The present invention relates to novel variants of the fluorescent protein GFP having improved fluorescence properties.

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### Background of the Invention

The use of Green Fluorescent Protein (GFP) derived from *Aequorea victoria* has revolutionised research into many cellular and molecular-biological processes. GFP allows researchers to label proteins within cells with an intrinsic fluor, so  
10 obviating the requirement to perform chemical labelling of proteins, and allowing development of assays of biological processes in intact living cells.

US 5491084 describes the use of GFP as a biological reporter. Early applications of GFP as a biological reporter (Chalfie et al. Science, (1994), 263, 802-  
15 5; Chalfie, et al. Photochem.Photobiol., (1995), 62(4), 651-6) used wild type (native) GFP (wtGFP), but these studies quickly demonstrated two areas of deficiency of wtGFP as a reporter for use in mammalian cells. Firstly, the protein being derived from a poikilothermic marine organism does not undergo protein folding efficiently when expressed in mammalian cells cultured at 37 °C, resulting in weak fluorescence.  
20 Secondly, the spectral characteristics of the wtGFP are not ideally suited to use as a cellular reporter, requiring excitation with electromagnetic radiation in the near-UV range, which is potentially damaging to living cells.

Consequently, significant effort has been expended to produce variant mutated forms of GFP with properties more suitable for use as an intracellular reporter.

A number of mutated forms of GFP with altered spectral properties have been  
5 described. A variant-GFP (Heim et al. (1994) Proc.Natl.Acad.Sci. 91, 12501)  
contains a Y66H mutation which blue-shifts the excitation and emission spectrum of  
the protein. However, this protein is only weakly fluorescent and requires potentially  
damaging UV excitation.

10 A further mutant of GFP (Heim et al. Nature. (1995), 373, 663-664) contains a  
S65T mutation which red-shifts the optimum excitation and emission wavelengths  
relative to wtGFP and which is 4-6 fold brighter than wtGFP when expressed as a  
recombinant protein at 25 °C. However, this variant does not yield bright  
fluorescence when expressed in hosts cultured at 37 °C.

15 Ehrig et al (FEBS Lett., (1995), 367,163-6) describe two mutations of GFP,  
T203I and E222G, which individually delete one of the excitation maxima of wtGFP.  
The E222G mutation deletes the near-UV excitation peak at 395 nm and produces a  
red-shift in the excitation peak at 475 nm to 481 nm. The emission peak for this  
20 mutant protein is at 506 nm.

WO96/27675 describes two variant GFPs, obtained by random mutagenesis  
and subsequent selection for brightness, which contain the mutations V163A and  
V163A+S175G, respectively. These variants were shown to produce more efficient

expression in plant cells relative to wtGFP and to increase the thermotolerance of protein folding. The double mutant V163A+S175G was observed to be brighter than the variant containing the single V163A mutant alone; however this mutant exhibits an undesirable blue-shifted excitation peak.

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A further mutant, termed cycle-3, generated by molecular evolution through DNA shuffling (Cramer, A. et al. *Nature Biotechnology*, (1996), 14, 315-9) is available commercially from Invitrogen Inc. Cycle-3-GFP contains three mutations (F99S+M153T+V163A) which increase whole cell fluorescence approximately 42 fold when compared with wtGFP. However, this mutant retains the near-UV excitation maximum of the wtGFP, making it less suitable as a reporter for use in living cells.

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The above mutations effectively address some of the spectral deficiencies of wtGFP as a biological reporter in providing variant forms of GFP which are compatible with lower energy excitation and which emit at wavelengths compatible with detection instrumentation commonly in use for measuring biological reporters. However, such mutations do not address the problem of inefficient folding and chromophore formation when wtGFP or spectral variants are expressed in hosts requiring growth at temperatures significantly greater than ambient.

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US 6172188 describes variant GFPs wherein the amino acid in position 1 preceding the chromophore has been mutated to provide an increase of fluorescence intensity. Such mutations include F64L, F64V, F64A, F64G and F64I, with F64I.

being the preferred mutation. These mutants result in a substantial increase in the intensity of fluorescence of GFP without shifting the excitation and emission maxima. F64L-GFP has been shown to yield an approximate 6-fold increase in fluorescence at 37 °C due to shorter chromophore maturation time.

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In addition to the single mutants or randomly derived combinations of mutations described above, a variety of mutant-GFPs have been created which contain two or more mutations deliberately selected from those described above and other mutations, and which seek to combine the advantageous properties of the individual  
10 mutations to produce a protein with expression and spectral properties which are suited to use as a sensitive biological reporter in mammalian cells.

One mutant, commonly termed EGFP, available commercially from Clontech Inc., contains the mutations F64L and S65T (Cormack, B.P. et al. *Gene*, (1996), 173,  
15 33-38). These mutations when combined, confer an approximate 35-fold increase in brightness over wtGFP and the spectral characteristics permit excitation and detection of EGFP with commonly used fluorescein excitation (488 nm) and emission filters (505 nm–530 nm). EGFP has been optimised for expression in mammalian systems, having been constructed with preferred mammalian codons.

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US 6194548 discloses GFPs with improved fluorescence and folding characteristics at 37 °C that contain, at least, the changes F64L and V163A and S175G. A further mutant GFP containing the F64L, S65T and V163A mutations has been described (Cubitt, A.B. et al. *Methods in Cell Biology*, (1999), 58, 19-29).

US 6077707 describes a blue fluorescent protein (BFP) containing the F64L mutation in combination with Y66H and US 6194548 describes a further BFP containing the F64L, Y66H, Y145F and L236R substitutions.

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### **Summary of the Invention**

In view of the needs of the prior art, the present invention provides novel engineered derivatives of green fluorescent protein (GFP) which have an amino acid sequence which is modified by amino acid substitution compared with the amino acid  
10 sequence of wild type Green Fluorescent Protein. The modified GFPs exhibit enhanced fluorescence relative to wtGFP when expressed in non-homologous cells at temperatures above 30 °C, and when excited at about 490 nm compared to the parent proteins, i.e. wtGFP. An example of a preferred protein is F64L-S175G-E222G-GFP. The modified GFPs provide a means for detecting GFP reporters in mammalian cells  
15 at lower levels of expression and/or increased sensitivity relative to wtGFP. This greatly improves the usefulness of fluorescent proteins in studying cellular functions in living cells.

The present invention provides a fluorescent protein which is derived from  
20 Green Fluorescent Protein (GFP), or any functional GFP analogue, and has an amino acid sequence which is modified by amino acid substitution as compared with the amino acid sequence of wild type Green Fluorescent Protein. The modified fluorescent protein includes an amino acid substitution at position F64, a single amino acid substitution at either position S65 or position E222, and an amino acid

substitution at position S175 whereby the modified GFP has a different excitation spectrum and or emission spectrum compared with wild type GFP.

Additionally, the present invention provides a fluorescent protein derived from  
5 Green Fluorescent Protein (GFP) and having the amino acid sequence as set forth in  
SEQ ID No.3 of Figure 3.

The present invention also provides a fluorescent protein derived from Green  
Fluorescent Protein (GFP) and having the amino acid sequence as set forth in SEQ ID  
10 No.4 of Figure 4.

The present invention further provides a nucleic acid molecule comprising a  
nucleotide sequence encoding a fluorescent protein which is derived from Green  
Fluorescent Protein (GFP), or any functional GFP analogue, and has an amino acid  
15 sequence which is modified by amino acid substitution compared with the amino acid  
sequence of wild type Green Fluorescent Protein. The modified fluorescent protein  
includes an amino acid substitution at position F64, a single amino acid substitution at  
either position S65 or position E222, and an amino acid substitution at position S175  
wherein the modified GFP has a different excitation spectrum and/or emission  
20 spectrum compared with wild type GFP.

The present invention also further provides a method of measuring the  
expression of a protein of interest in a cell. The method includes the steps of i)  
introducing into a cell a nucleic acid molecule comprising a nucleotide sequence

encoding a fluorescent protein which is derived from Green Fluorescent Protein (GFP), or any functional GFP analogue, according to the present invention. The nucleic acid molecule is operably linked to and under the control of an expression control sequence which moderates expression of said protein of interest: ii) culturing  
5 the cell under conditions suitable for the expression of the protein of interest: and iii) detecting the fluorescence emission of the Green Fluorescent Protein (GFP) or a functional GFP analogue as a means of measuring the expression of the protein of interest.

10 The present invention still further provides a method of determining the cellular and/or extracellular localisation of a protein of interest. The method includes the steps of i) introducing into a cell a nucleic acid molecule having a nucleotide sequence encoding a fluorescent protein which is derived from Green Fluorescent Protein (GFP), or any functional GFP analogue, according to the present invention  
15 and fused to a nucleotide sequence encoding a protein of interest. The nucleic acid molecule is operably linked to and under the control of a suitable expression control sequence: ii) culturing the cell under conditions suitable for the expression of the protein of interest: and iii) determining the cellular and/or extracellular localisation of the protein of interest by detecting the fluorescence emission by optical means.

20 The present invention even still further provides a method of comparing the effect of one or more test substance(s) on the expression and/or localisation of one or more different protein(s) of interest in a cell. The method includes the steps of i) introducing into a cell a nucleic acid molecule comprising a nucleotide sequence

encoding a Green Fluorescent Protein (GFP) or a functional GFP analogue according to the present invention and optionally fused to a nucleotide sequence encoding a first protein of interest, where the nucleic acid molecule is operably linked to and under the control of a first expression control sequence: ii) culturing the cells under  
5 conditions suitable for the expression of the protein(s) of interest in the presence and absence of the test substance(s): iii) determining the expression and/or localisation of the protein(s) of interest in the cells by detecting the fluorescence emission by optical means: and iv) comparing the fluorescence emission obtained in the presence and absence of the test substance(s) to determine the effect of the test substance(s) on the  
10 expression and/or localisation of the protein(s) of interest. The introducing step may also include at least one different nucleic acid molecule encoding a protein reporter molecule optionally fused to a different protein of interest where each nucleic acid molecule is operably linked to and under the control of a second expression control sequence wherein the protein reporter molecule has or is capable of generating an  
15 emission signal which is spectrally distinct from that of the Green Fluorescent Protein (GFP) or functional GFP analogue.

### **Brief Description of the Drawings**

**Figure 1** is the nucleotide Sequence of wtGFP (Chalfie et al. Science. (1994).  
20 263, 802-5) and referred to herein as SEQ ID No.1.

**Figure 2** is the corresponding amino acid sequence of wtGFP (Chalfie et al. Science. (1994). 263, 802-5) and referred to herein as SEQ ID No.2.

**Figure 3** is the predicted amino acid sequence of F64L-S175G-E222G-GFP and referred to herein as SEQ ID No.3.

**Figure 4** is the predicted amino acid sequence of F64L-S65T-S175G-GFP and referred to herein as SEQ ID No.4.

**Figure 5** is a plot showing average fluorescence intensities of mutant GFPs according to the invention.

5        **Figure 6** is a plot showing relative photodegradation of mutant GFPs according to the invention.

**Figure 7** is a plot demonstrating the increase in the ratio of nuclear to cytoplasmic fluorescence intensity on translocation of P65-GFP from the cytoplasm to the nucleus of CHO-hir cells following agonist addition.

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#### **Detailed Description of the Preferred Embodiments**

The present invention provides novel engineered derivatives of green fluorescent protein (GFP) which exhibit enhanced fluorescence relative to wtGFP when expressed in non-homologous cells at temperatures above 30°C, and when  
15        excited at about 490 nm compared to the parent proteins, i.e. wtGFP. Mutant GFPs according to the invention provide a means for detecting GFP reporters in mammalian cells at lower levels of expression and/or increased sensitivity relative to wtGFP. This greatly improves the usefulness of fluorescent proteins in studying cellular functions in living cells. The multiply-mutated GFPs of this invention have fluorescence  
20        properties which are not predictable from the properties of the individual mutations when studied in isolation. Furthermore, it has surprisingly been found that certain GFPs according to the present invention, which do not contain any mutations in the chromophore region relative to wtGFP, exhibit enhanced fluorescence compared with mutant GFPs described previously.

In a first aspect of the invention, there is provided a fluorescent protein which is derived from Green Fluorescent Protein (GFP) or any functional GFP analogue and has an amino acid sequence which is modified by amino acid substitution compared  
5 with the amino acid sequence of wild type Green Fluorescent Protein said modified fluorescent protein comprising:

- i) an amino acid substitution at position F64;
  - ii) a single amino acid substitution at a position selected from the group consisting of positions S65 and F222; and
  - 10 iii) an amino acid substitution at position S175;
- wherein the modified GFP has a different excitation spectrum and/or emission spectrum compared with wild type GFP.

Suitably, the amino acid F at position 64 may be substituted by an amino acid  
15 selected from the group consisting of L, I, V, A and G, thereby providing F64L, F64I, F64V, F64A, or F64G substitutions. In a preferred embodiment of the first aspect, the amino acid F is substituted by L at position 64.

Suitably, the amino acid S at position 175 may be substituted by an amino acid  
20 selected from the group consisting of G, A, L, I and T, thereby providing S175G, S175A, S175L, S175I and S175T substitutions. In a preferred embodiment of the first aspect, the amino acid S is substituted by G at position 175.

In embodiments where the amino acid S at position 65 is substituted, it is suitably substituted by an amino acid selected from the group consisting of G, A, L, C, V, I and T, thereby providing S65G, S65A, S65L, S65C, S65V, S65I or S65T substitutions. Preferably, the amino acid substitution at position 65 is the S65T substitution.

In embodiments where the amino acid E at position 222 is substituted, it is suitably substituted by an amino acid selected from the group consisting of G, A, V, L, I, F, S, T, N and Q, thereby providing E222G, E222A, E222V, E222L, E222I, E222F, E222S, E222T, E222N or E222Q substitutions. Preferably, the amino acid substitution at position 222 is the E222G substitution.

Suitably, the novel fluorescent proteins exhibit high fluorescence in cells expressing them when said cells are incubated at a temperature of 30 °C or above, preferably at a temperature of from 32 °C to 39 °C, more preferably from 35 °C to 38 °C and most preferably at a temperature of about 37 °C.

Preferably, the fluorescent protein according to the first aspect has an amino acid sequence which is modified by amino acid substitution compared with the amino acid sequence of wild type Green Fluorescent Protein having the sequence: SEQ ID No.2.

A preferred protein according to the present invention is a protein in which, in relation to SEQ ID No.2 of GFP, the amino acid F at position 64 has been substituted

by L, the amino acid S at position 175 has been substituted by G and the amino acid E at position 222 has been substituted by G, and is shown herein as having the amino acid sequence as set forth in SEQ ID No.3.

5           An alternative preferred protein according to the present invention is a protein in which, in relation to SEQ ID No.2 of GFP, the amino acid F at position 64 has been substituted by L, the amino acid S at position 65 has been substituted by T and the amino acid S at position 175 has been substituted by G, and is shown herein as having the amino acid sequence as set forth in SEQ ID No.4.

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Suitably, the GFP or functional GFP-analogue used to derive the fluorescent protein may be obtained from any convenient source. For example, native GFP derived from species of the genus *Aequorea*, suitably *Aequorea victoria*. The chromophore in wtGFP from *Aequorea victoria* is at positions 65-67 of the predicted primary amino acid sequence (SEQ ID No.2). In a preferred embodiment, the GFP is  
15           derived from *Aequorea victoria*.

The modified proteins of the present invention may be produced by introducing mutations in a sequence of the nucleic acid that encodes the protein. As  
20           used herein, a preferred sequence of the gene encoding wtGFP is derived from *Aequorea victoria*, published by Chalfie et al. (Science, (1994), 263, 802-5) disclosed as SEQ ID No.1 (Figure 1). The corresponding amino acid sequence is shown in SEQ ID No.2 (Figure 2). Alternative sequences of the GFP gene may be used, for example, the nucleotide (and predicted amino acid) sequences of the GFP gene

described by Prasher et al. (Gene (1992), 111, 229) and the sequences as disclosed in WO 97 11094. In addition, alternative gene sequences that encode the fluorescent protein may incorporate a consensus Kozak nucleotide sequence (Kozak, M., Cell (1986), 44, 283), or preferred mammalian codons, to provide improved translation in mammalian systems. The nucleotide sequence corresponding to the fluorescent protein may also encode useful restriction enzyme sites and additional elements such as target sites for enzymes and purification tags. Methods for incorporation of a Kozak region, preferred mammalian codons, restriction enzyme sites, enzyme sites and purification tags are well known in the art and may result in the incorporation of amino acid residues and a change in numbering of amino acid residues in the fluorescent protein relative to the wtGFP numbering in the sequence provided.

Herein, the abbreviations used for the amino acids are those stated in J.Biol.Chem., (1968), 243, 3558.

In a second aspect of the invention, there is provided a fusion compound comprising a protein of interest fused to a fluorescent protein which is derived from Green Fluorescent Protein (GFP) or any functional GFP analogue and has an amino acid sequence which is modified by amino acid substitution compared with the amino acid sequence of wild type Green Fluorescent Protein said modified fluorescent protein comprising:

- i) an amino acid substitution at position F64;
- ii) a single amino acid substitution at a position selected from the group consisting of positions S65 and E222; and

iii) an amino acid substitution at position S175;

wherein said modified GFP has a different excitation spectrum and/or emission spectrum compared with wild type GFP.

5 In the context of the present invention, the term "protein of interest" is intended also to encompass polypeptides and peptide fragments. Examples of such proteins of interest include: NF $\kappa$ B and subunits thereof, RAC1, PLC domains, MAPKAP2, PKC, Cytochrome C, RHO,  $\beta$ -actin, STAT6, protein kinase C isotypes, LAMP1/2 TGN, ATP7A TGN and GLUT4.

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In a third aspect of the present invention there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a fluorescent protein which is derived from Green Fluorescent Protein (GFP) or any functional GFP analogue and has an amino acid sequence which is modified by amino acid substitution compared  
15 with the amino acid sequence of wild type Green Fluorescent Protein said modified fluorescent protein comprising:

i) an amino acid substitution at position F64;

ii) a single amino acid substitution at a position selected from the group consisting of positions S65 and E222; and

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iii) an amino acid substitution at position S175;

wherein said modified GFP has a different excitation spectrum and/or emission spectrum compared with wild type GFP.

Preferably, the nucleic acid molecule according to the third aspect encodes a fluorescent protein having an amino acid sequence which is modified by amino acid substitution compared with the amino acid sequence of wild type Green Fluorescent Protein having the sequence: SEQ ID No.2.

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In a particular embodiment of the third aspect, the nucleic acid molecule comprises a nucleotide sequence encoding a fluorescent protein derived from Green Fluorescent Protein (GFP) or any functional GFP analogue according to the invention fused to a nucleotide sequence encoding a protein of interest.

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Preferably, the nucleic acid molecule is a construct comprising a DNA sequence.

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Preferably, the nucleic acid molecule encodes a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID No.3 and SEQ ID No.4.

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As is well known, a single amino acid may be encoded by more than one nucleotide codon and thus each of the above nucleotide sequences may be modified to produce an alternative nucleotide sequence that encodes the same peptide. Thus, the preferred embodiments of the invention include alternative DNA sequences that encode the preferred proteins as previously described. It is to be understood that the preferred proteins (and the nucleic acid sequences from which they are derived), may

include additional residues, particularly N- and C-terminal amino acids, or 5'- or 3'- nucleotide sequences, and still be essentially as described herein.

Suitably, the DNA construct encoding the novel fluorescent proteins may be  
5 prepared synthetically by established methods, e.g. the phosphoramidite method  
described by Beaucage and Caruthers, (Tetrahedron Letters (1981), 22, 1859-1869),  
or the method described by Matthes et al., (EMBO Journal (1984), 3, 801-805).  
According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an  
automatic DNA synthesizer, purified, annealed, ligated and cloned into suitable  
10 vectors.

The DNA construct encoding the fluorescent protein may also be prepared by  
recombinant DNA methodology, for example cDNA cloning. See for example,  
Sambrook, J. et al (1989) Molecular Cloning - A Laboratory Manual, Cold Spring  
15 Harbor Laboratory Press.

The DNA construct may also be prepared by polymerase chain reaction (PCR)  
using specific primers, for instance as described in US 4683202 or by Saiki et al  
(Science (1988), 239, 487-491). A recent review of PCR methods may be found in  
20 PCR Protocols, (1990), Academic Press, San Diego, California, USA.

The gene sequence encoding the fluorescent protein may be joined in-frame  
with a gene encoding the protein of interest and the desired fusion protein produced  
when inserted into an appropriate expression vector. For example, polymerase chain

reaction or complementary oligonucleotides may be employed to engineer a polynucleotide sequence corresponding to the fluorescent protein, 5' or 3' to the gene sequence corresponding to the protein of interest. Alternatively, the same techniques may be used to engineer a polynucleotide sequence corresponding to the fluorescent protein sequence 5' or 3' to the multiple cloning site of an expression vector prior to insertion of a gene sequence encoding the protein of interest. The polynucleotide sequence corresponding to the fluorescent protein sequence may comprise additional nucleotide sequences to include cloning sites, linkers, transcription and translation initiation and/or termination signals, labelling and purification tags.

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In a fourth aspect, there is provided an expression vector comprising suitable expression control sequences operably linked to a nucleic acid molecule according to the present invention. The DNA construct of the invention may be inserted into a recombinant vector, which may be any vector that may conveniently be subjected to recombinant DNA procedures. The choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, ie. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, eg. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

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The vector is preferably an expression vector in which the DNA sequence encoding a fluorescent protein of the invention is operably linked to additional

segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and  
5 proceeds through the DNA sequence coding for the fluorescent protein of the invention.

The promoter may be any DNA sequence which shows transcriptional activity in a suitable host cell of choice, (eg. a bacterial cell, a mammalian cell, a yeast cell, or  
10 an insect cell) for expressing a fluorescent protein. The promoter may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding the fluorescent protein of the invention in mammalian cells are the  
15 CMV promoter (US 5168062, US5385839), Ubiquitin C promoter (Wulff, M. et al., FEBS Lett. (1990), 261, 101-105), SV40 promoter (Subramani et al., Mol. Cell Biol. (1981), 1, 854-864) and MT-1 (metallothionein gene) promoter (Palmiter et al., Science (1983), 222, 809-814). An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4745051; Vasuvedan et al., FEBS Lett., (1992)  
20 311, 7-11). Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem., (1980), 255, 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen., (1982), 1, 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for

Chemicals (Hollaender et al. eds.), Plenum Press, New York, 1982), or the TPI1 (US 4599311) or ADH2-4c (Russell et al., Nature, (1983), 304, 652-654) promoters.

5        Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylosidase gene, or the phage Lambda PR or PL promoters or the *Escherichia coli* *lac*, *trp* or *tac* promoters.

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      The DNA sequence encoding the novel fluorescent proteins of the invention may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators.

15        The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

20        The vector may further comprise a DNA sequence enabling internal ribosomal entry and expression of two proteins from one bicistronic transcript mRNA molecule. For example, the internal ribosomal entry sequence from the encephalomyocarditis virus (Rees S. et al, BioTechniques (1996), 20, 102-110 and US 4937190).

The recombinant vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

5           When the host cell is a yeast cell, examples of suitable sequences enabling the vector to replicate are the yeast plasmid 2 $\mu$  replication genes REP 1-3 and origin of replication.

10           The vector may also comprise selectable markers, such as a gene that confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, puromycin, neomycin or hygromycin.

15           The procedures used to ligate the DNA sequences coding for the fluorescent protein of the invention, the promoter and optionally the terminator and/ or targeting sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (eg. Sambrook et al., op.cit.).

20           In a fifth aspect of the invention, there is provided a host cell transformed or transfected with a DNA construct comprising an expression vector according to the present invention.

          The DNA construct or the recombinant vector of the invention is suitably introduced into a host cell which may be any cell which is capable of expressing the

present DNA construct and includes bacteria, yeast and higher eukaryotic cells (Unger, T.F., The Scientist (1997), 11(17), 20-23; Smith, C., The Scientist (1998), 12(22): 20; Smith, C., The Scientist (1998), 12(3), 18; Fernandez, J.M. & Hoefler, J.P., Gene Expression Systems- using nature for the art of expression, Academic Press  
5 1999).

Examples of bacterial host cells which, on cultivation, are capable of expressing the DNA construct of the invention are Gram-positive bacteria, eg. species of *Bacillus* or Gram-negative bacteria such as *E. coli*. The transformation of the  
10 bacteria may be effected by using competent cells in a manner known per se (cf. Sambrook et al., supra).

Examples of suitable mammalian cell lines are the HEK293 and the HeLa cell lines, primary cells, and the COS (e.g. ATCC CRL 1650), BHK (eg. ATCC CRL  
15 1632, ATCC CCL 10), CHL (e.g. ATCC CCL39) or CHO (eg. ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in eg. Kaufman and Sharp, J. Mol. Biol., (1982), 159, 601-621; Southern and Berg, J. Mol. Appl. Genet., (1982), 1, 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA, (1982), 79, 422-426; Wigler et al., Cell, (1978), 14,  
20 725; Corsaro and Pearson, Somatic Cell Genetics, (1981), 7, 603, Graham and van der Eb, Virology (1973), 52, 456; and Neumann et al., EMBO J., (1982), 1, 841-845.

Examples of suitable yeast cells include cells of *Saccharomyces* spp. or *Schizosaccharomyces* spp., in particular strains of *Saccharomyces cerevisiae* or

*Saccharomyces kluyveri*. Methods for transforming yeast cells with heterologous DNA and producing heterologous polypeptides therefrom are described, e.g. in US 4599311, US 4931373, US 4870008, US 5037743, and US 4845075, all of which are hereby incorporated by reference. Transformed cells are selected by a phenotype  
5 determined by a selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient, e.g. leucine. A preferred vector for use in yeast is the POT1 vector disclosed in US 4931373. The DNA sequence encoding the fluorescent protein of the invention may be preceded by a signal sequence and optionally a leader sequence, e.g. as described above. Further examples of suitable  
10 yeast cells are strains of *Kluyveromyces*, such as *K. lactis*, *Hansenula*, e.g. *H. polymorpha*, or *Pichia*, e.g. *P. pastoris* (cf. Gleeson et al., J. Gen. Microbiol., (1986), 132, 3459-3465; US 4882279).

Transformation of insect cells and production of heterologous polypeptides  
15 therein may be performed as described in US 4745051; US 4879236; US 5155037; US 5162222; EP 397485, all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5077214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO  
20 89/01028, or any of the aforementioned references.

In a sixth aspect, the invention provides a method for preparing a Green Fluorescent Protein (GFP) or a functional GFP analogue according to the present invention, the method comprising cultivating a host cell transformed or transfected

with a nucleotide sequence according to the invention and obtaining therefrom the polypeptide expressed by said nucleotide sequence.

Suitably, the transformed or transfected host cells as described above are  
5 cultured in a suitable nutrient medium under conditions permitting the expression of a DNA construct according to the invention, after which the cells may be used in the screening method of the invention. Alternatively, the cells may be disrupted after which cell extracts and/or supernatants may be analysed for fluorescence and/ or used to purify the GFP or functional GFP analogue of the invention.

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The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published protocols (eg. in catalogues of the American  
15 Type Culture Collection: Sambrook et al., supra).

For example, a fusion protein comprising glutathione S-transferase (GST) and GFP can be constructed and expressed in *E. coli*. The GFP may be joined in-frame to the C-terminus of GST in a pGEX plasmid vector (Amersham Pharmacia Biotech).  
20 Recombinant production of the fusion protein is carried out utilising a standard *E. coli* expression host, followed by purification employing glutathione affinity chromatography and removal of the GST tag by proteolytic cleavage.

In a seventh aspect of the present invention, there is provided a method of measuring the expression of a protein of interest in a cell. The method comprises:

- i) introducing into a cell a nucleic acid molecule comprising a nucleotide sequence encoding a fluorescent protein which is derived from Green Fluorescent Protein (GFP) or any functional GFP analogue according to the present invention said nucleic acid molecule being operably linked to and under the control of an expression control sequence which moderates expression of said protein of interest;
- ii) culturing the cell under conditions suitable for the expression of the protein of interest; and
- iii) detecting the fluorescence emission of the Green Fluorescent Protein (GFP) or a functional GFP analogue as a means of measuring the expression of the protein of interest.

In an eighth aspect of the present invention, there is provided a method of determining the cellular and/or extracellular localisation of a protein of interest which method comprises:

- i) introducing into a cell a nucleic acid molecule comprising a nucleotide sequence encoding a Green Fluorescent Protein (GFP) or a functional GFP analogue according to the invention fused to a nucleotide sequence encoding a protein of interest, said nucleic acid molecule being operably linked to and under the control of a suitable expression control sequence;

- ii) culturing said cell under conditions suitable for the expression of said protein of interest; and
- iii) determining the cellular and or extracellular localisation of said protein of interest by detecting the fluorescence emission by optical means.

5

The fluorescent proteins of the present invention may also be used in a method to detect and compare the effect of a test substance on the regulation of expression and/or translocation of two or more different proteins of interest in a cell.

Alternatively, they may be used in a method to compare the expression of a protein of  
10 interest and the simultaneous activity of an expression control sequence in response to a test substance. The fluorescent proteins may also be used in a method to compare the activity of two or more expression control sequences in a cell in response to a test substance. Such methods may be performed in the presence and in the absence of a test substance whose effect on the process is to be measured. For example, one  
15 detectable reporter molecule may be used as an internal reference and another as a variable marker, since regulated expression of a gene can be monitored quantitatively by fusion of an expression control sequence to a DNA construct encoding, eg. F64L-S175G-E222G-GFP, measuring the fluorescence, and normalising it to the fluorescence of a constitutively expressed spectrally distinct fluorescent molecule.  
20 The constitutively expressed spectrally distinct fluorescent molecule, for example BFP, acts as an internal reference.

Thus, in a ninth aspect of the present invention, there is provided a method of comparing the effect of one or more test substance(s) on the expression and/or

localisation of one or more different protein(s) of interest in a cell which method comprises:

i) introducing into a cell:

a) a nucleic acid molecule comprising a nucleotide sequence encoding a Green Fluorescent Protein (GFP) or a functional GFP analogue according to the invention optionally fused to a nucleotide sequence encoding a first protein of interest, said nucleic acid molecule being operably linked to and under the control of a first expression control sequence; and optionally,

b) at least one different nucleic acid molecule encoding a protein reporter molecule optionally fused to a different protein of interest, each said nucleic acid molecule being operably linked to and under the control of a second expression control sequence wherein said protein reporter molecule has or is capable of generating an emission signal which is spectrally distinct from that of said Green Fluorescent Protein (GFP) or a functional GFP analogue;

ii) culturing said cells under conditions suitable for the expression of said protein(s) of interest in the presence and absence of said test substance(s);

iii) determining the expression and/or localisation of said protein(s) of interest in said cells by detecting the fluorescence emission by optical means; and

iv) comparing the fluorescence emission obtained in the presence and absence of said test substance(s) to determine the effect of said test

substance(s) on the expression and/or localisation of said protein(s) of interest.

In a preferred embodiment of the ninth aspect, samples of said cells in a fluid  
5 medium are introduced into separate vessels for each of said test substances to be studied.

Preferably, the first and second expression control sequences are different.

10 Suitably, the protein reporter molecule may be selected from the group consisting of fluorescent proteins and enzymes. Preferred fluorescent proteins are those which have a spectrally distinguishable emission wavelength compared with the emission wavelength of the fluorescent proteins according to the present invention, for example, BFP. Suitable enzyme reporters are those which are suitable for  
15 generating a detectable (eg. a luminescent or fluorescent) signal in a substrate. Suitable enzyme/substrates include: luciferase/luciferin;  $\beta$ -galactosidase/DDAO galactoside;  $\beta$ -galactosidase/fluorescein di- $\beta$ -D-galactopyranoside; alkaline phosphatase/Attphos.

20 In the methods of the invention, the fluorescence of cells transformed or transfected with the DNA construct according to the invention may suitably be measured by optical means in for example: a spectrophotometer, a fluorimeter, a fluorescence microscope, a cooled charge-coupled device (CCD) imager (such as a scanning imager or an area imager), a fluorescence activated cell sorter, a confocal

microscope or a scanning confocal device, where the spectral properties of the cells in culture may be determined as scans of light excitation and emission.

The fluorescent proteins of the present invention have many additional  
5 applications, for example:

- 10 i) Use as a non-toxic marker for selection of transfected cells containing an expression vector encoding at least the fluorescent protein of the invention. The fluorescent emission may be used to isolate transfected cells thereby overcoming the need for selection with toxic molecules such as antibiotics.
- 15 ii) Use as a protein label in living and fixed cells. The novel proteins exhibit strong fluorescence and are a suitable label for proteins present at low concentrations. Since no substrate is needed and visualization of the fluorescent protein does not damage the cells, dynamic analysis can be performed.
- 20 iii) Use as a marker in cell or organelle fusion. By labelling one or more cells or organelles with the novel proteins, for example, F64L-S175G-E222G-GFP, and other cells or organelles with same or another fluor, fusions such as heterokaryon formation can be monitored.
- iv) Translocation of proteins fused to the novel proteins of the invention can be visualised. The translocation of intracellular proteins to a specific organelle can be visualised by fusing the protein of interest to a fluorescent protein, for example, F64L-S175G-E222G-GFP and labelling the organelle with another fluorescent molecule, eg.

fluorescent protein. Translocation can then be detected as a spectral shift in the fluorescent proteins in the specific organelle.

- v) Use as a secretion marker. By fusion of a fluorescent protein of the invention to a signal peptide or a peptide to be secreted, secretion may be followed in living cells.
- vi) Use as genetic reporter or protein tag in transgenic animals. Due to the strong fluorescence of the novel proteins, they are suitable as tags for proteins and gene expression, since the signal to noise ratio is significantly improved over the prior art proteins, such as wild-type GFP.
- vii) Use as a cell or organelle integrity marker. By expressing the novel proteins targeted to an organelle, it is possible to calculate the leakage of the protein and use that as a measure of cell integrity.
- viii) Use as a transfection marker, and as a marker to be used in combination with FACS sorting (eg. as described in Example 3). Due to the increased brightness of the novel proteins the quality of cell detection and sorting can be significantly improved.
- ix) Use as real-time probe working at near physiological concentrations. Since the novel proteins of the present invention are significantly brighter than wtGFP when expressed in cells at about 37 °C and excited with light at about 490 nm, the concentration needed for visualization can be lowered. Target sites for enzymes engineered into the novel proteins, for example F64L-S175G-E222G-GFP, can therefore be present in the cell at low concentrations in living cells.

This is important for two reasons: i) the probe must interfere as little as possible with the intracellular process being studied; and ii) the translational and transcriptional apparatus should be stressed minimally.

- 5           x)    Transposon vector mutagenesis can be performed using the novel proteins as markers in transcriptional and translational fusions. Transposons may be used in microorganisms encoding the novel proteins. The transposons may be constructed for translational and transcriptional fusion to be used for screening for promoters.
- 10           Transposon vectors encoding the novel proteins, for example F64L-S175G-E222G-GFP, can be used for tagging plasmids and chromosomes.
- 15           xi)   Use as a reporter for bacterial detection by introducing the novel proteins into the genome of bacteriophages. By engineering the novel proteins, for example F64L-S175G-E222G-GFP, into the genome of a phage a diagnostic tool can be designed. F64L-S175G-E222G-GFP will be expressed only upon transfection of the genome into a living host. The host specificity is defined by the bacteriophage.

20

## EXAMPLES

### 1. Cloning of GFP gene and template vector construction

5           The GFP gene used in the present study was contained within the plasmid pGFP (Chalfie et al., Science, (1994), 263, 802-805; GenBank accession number U17997) obtained from Clontech Laboratories Inc. (Palo Alto, Ca, USA). The gene was amplified by PCR using Pfu polymerase (Promega, Madison, WI, USA) according to recognised protocols (Saiki et al., Science, (1988), 239, 487-491). The  
10 sequences of primers used were:

GFP-1	5'-ggtaacgggcccaccatgagtaaaggagaagaacttttcac	SEQ ID No.5
GFP-2	5'-ggtaacgggttaaccggtttgtatagttcaccatg	SEQ ID No.6
GFP-3	5'-ggtaacgggcccaccatgggatccaaaggagaagaacttttcac	SEQ ID No.7

Primer GFP-1 exhibits homology to the 5' region of the GFP gene and contains a partial Kozak site (Kozak, M. Cell, (1986), 44, 283) prior to the start codon  
15 for efficient initiation of translation in mammalian systems. Primer GFP-2 exhibits homology to the 3' region of the GFP gene and contains an additional *AgeI* restriction enzyme site immediately prior to the stop codon to facilitate cloning of proteins by fusion to the C-terminus of GFP. Primer GFP-3 is similar to primer GFP-1 exhibiting homology to the 5' region of the GFP gene, but contains an additional restriction site  
20 (*Bam*III) immediately after the initiation codon to facilitate cloning of proteins by fusion to the N-terminus of GFP. Amplified products resulting from PCR reactions

containing primers GFP-1 and GFP-2, and GFP-3 and GFP-2 were tailed with a single 3'-deoxyadenosine using Taq polymerase (Amersham Pharmacia Biotech, Amersham, UK) and ligated into the TA cloning vector pTARGET (Promega) according to manufacturer's instructions. The correct orientation relative to the CMV promoter and sequence of the insert was determined by automated DNA sequencing.

## 2. Generation of mutants of GFP

The following mutants of GFP were generated in the present study: F64L-GFP, V163A-GFP, S175G-GFP, E222G-GFP, F64L-E222G-GFP, F64L-V163A-GFP, F64L-S175G-GFP, V163A-S175G-GFP, V163A-E222G-GFP, S175G-E222G-GFP, F64L-S175G-E222G-GFP, V163A-S175G-E222G-GFP, F64L-V163A-E222G-GFP, F64L-S65T-S175G-GFP, F64L-S65T-V163A-GFP. Mutants of the GFP gene (SEQ ID 3) construct within pTARGET (See Example 1) were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, Ca, USA) according to manufacturer's instructions. The sequences of primers used to generate F64L, S65T, V163A, S175G and E222G single mutants have been documented in Table 1. Multiply-mutated GFP molecules were generated through successive mutagenesis reactions. All GFP mutant sequences were verified by automated sequencing.

Table 1

<u>Primer</u>	<u>Mutation</u>	<u>Sequence (5' - 3')</u>	<u>SEQ</u> <u>ID No.</u>
GFP-64f	F64L	ccaacacttgctactactctcttattggtgttcaat	8
GFP-64r	F64L	attgaacaccataagagagagtagtgacaagtgttgg	9
GFP-65f	S65T	ccaacacttgctactactctcaactatgggtgttcaatgctttca	10
GFP-65r	S65T	tgaaaagcattgaacaccatagggtgagagtagtgacaagtgttgg	11
GFP-163f	V163A	gacaaacaaaagaatggaatcaaagccaacttcaaaattagacac	12
GFP-163r	V163A	gtgtctaatttgaagttggctttgatccatttttgtttgtc	13
GFP-175f	S175G	caacattgaagatggaggcggtcaactagcagacc	14
GFP-175r	S175G	ggctctgctagttgaacgcctccatcttcaatgttg	15
GFP-222f	E222G	ccacatggctccttcttggctttgtaacagctgctgg	16
GFP-222r	E222G	ccagcagctgttacaaagccaagaaggaccatgtgg	17

3. Influence of individual mutations and combinations of F64L, S65T, V163A,  
5 S175G and E222G mutations upon GFP when expressed in mammalian cells

Plasmid DNA to be used for transfection was prepared for all GFP and EGFP constructs using the HiSpeed plasmid purification kit (Qiagen, Westberg, NL). DNA was diluted to 100 ng.  $\mu$ l<sup>-1</sup> in 18-Megohm water (Sigma) and 1  $\mu$ g used for  
10 transfections. For 50-80% confluency on the day of transfection, HeLa cells were plated at a density of  $5 \times 10^4$ /well in 6-well plates and incubated overnight. A 1:3 (1  $\mu$ g : 3  $\mu$ l) ratio of DNA to FuGene6 reagent (Roche) was used for each transient

transfection reaction: 3 µl FuGene6 was added to 87 µl serum-free DMEM medium (Sigma) (containing penicillin streptomycin, L-glutamine (GibcoBRL) and gently tapped to mix, then 10 µl (1 µg) construct DNA was added and again gently mixed. The FuGene6:DNA complex was incubated at room temperature for 40 minutes then  
5 added dropwise directly to the cells without changing the medium, and the plates swirled for even distribution.

Fluorescence measurements were made 24 or 48 hours after transfection. Briefly, the cells were washed in phosphate-buffered saline, released with the addition  
10 of 2 drops of Trypsin (GibcoBRL) and resuspended in 1 ml of complete DMEM medium (containing penicillin/streptomycin, L-glutamine and foetal bovine serum (Sigma). The cells were vortexed and analysed on a FACS Calibur flow cytometer (Becton Dickinson & Co., NJ, USA) for characterisation of whole cell fluorescence, with excitation at 488 nm and emission viewed with fluorescence filter set 530/30nm  
15 (range 515-545 nm). 10,000 events were collected for each transfection and 6-10 replicates carried out for each construct. Average fluorescent intensities from the FACS analysis were obtained as geometric means (mean fluorescence on log scale) and are shown in Figure 5.

20

#### 4. Purification of fluorescent proteins from *E. coli*

The gene for the mutant F64L-S175G-E222G-GFP (Example 2) was excised from pTARGET with *Bam*HI and *Sal*II and sub-cloned into the IPTG-inducible, GST-

fusion vector pGEX-6P1 (Amersham Pharmacia Biotech). *E. coli* JM109 cells (Promega) containing an expression vector with the GST-GFP gene fusion were grown at 30°C to an  $OD_{600}=0.6$  in 2x YT broth containing 100 µg/ml ampicillin. Protein expression was induced with IPTG (0.1 mM) and incubation continued for 16  
5 hours. Cells were pelleted by centrifugation, resuspended in PBS and lysed by sonication (four 10 second bursts at 20 µm with intermittent cooling on ice). Cellular debris was removed by centrifugation and the lysate containing soluble GST-GFP fusion protein was purified using glutathione sepharose columns (Amersham Pharmacia Biotech). Protein was then exchanged and eluted in PBS using a PD10  
10 column (Amersham Pharmacia Biotech). The presence of a single band of correct molecular weight in the protein preparation was confirmed by SDS-PAGE using 4-12% Bis-Tris NuPAGE gel electrophoresis (Invitrogen). To assess protein concentration and purity, the protein preparation was subjected, in duplicate, to acid hydrolysis and filtration before amino acid analysis by ion exchange chromatography  
15 using a Pharmacia alpha plus series II analyser.

The extinction coefficient (Table 2) was determined on a UV/vis spectrometer (Unicam). Quantum yield (Table 2) was determined according to the method documented by Patterson et al (Biophysical Journal, (1997), 73, 2782-2790). Samples  
20 of equal optical density at respective absorbance maxima were prepared, and diluted, in 10mM Tris.HCl pH 8 for the purified GFP preparation and a fluorescein reference standard (Molecular Probes). Fluorescence emission was measured in the region 490 – 600nm using a LS50B luminescence spectrometer (Perkin Elmer) and results for the

GFP preparation were compared directly to those for the fluorescein standard (QY=0.85).

Table 2

<u>Protein</u>	<u>Absorbance</u> peak (nm)	<u>Extinction coefficient</u> (M <sup>-1</sup> cm <sup>-1</sup> )	<u>Emission</u> peak (nm)	<u>QY</u>
F64L-S175G-E222G-GFP	481	46213*	506	0.6*

\*Mean of two measurements

To evaluate the degree of photodegradation of the mutants F64L-S175G-E222G-GFP and F64L-E222G relative to wtGFP, 50ng of DNA was transfected into HeLa cells according to the method outlined in Example 3. For 50-80% confluency on the day of transfection, HeLa cells were plated at a density of  $5 \times 10^3$ /well in a ViewPlate<sup>TM</sup>-96 (Packard, Meriden CT, USA). Twenty-four hours after transfection, the cells were imaged live on a LEADseeker<sup>TM</sup> Cell Analysis System (Amersham Pharmacia Biotech) and bleached at high laser power (19.94mW) with a 488nm Argon laser (emission filter 535-45nm). Thirty-two individual images were taken over 260s with non-continuous illumination and all fluorescent proteins showed marked photodegradation as shown in Figure 6.

5. Measurement of NFκB translocation

NFκB is an activator of transcription and a component of signalling pathways which are responsive to a variety of inducers including cytokines, lymphokines, and  
5 some immunosuppressive agents.

The human NFκB P65 subunit gene (GenBank Accession number: M62399) was amplified using PCR according to recognised protocols (Saiki et al., Science, (1988), 239, 487-491). The sequences of primers used were:

10

NFκB-1 5'-ttttactcgagatggacgaactgttccccctca

SEQ ID No.18

NFκB-2 5'-ttttgaagcttggagctgactgcagcagg

SEQ ID No.19

15

The P65 subunit was fused to the N terminus of GFP (SEQ ID No.3) in the vector pCORON1000 (Amersham Pharmacia Biotech), under the control of a CMV promoter. This was transfected into CHO-hir cells using FuGene6 reagent (Roche) and standard transfection procedures and a stable cell line was produced containing  
the P65-GFP construct.

CHO-hir, P65-GFP cells were seeded into 96 well microtitre plates at a confluency of  $5 \times 10^3$  cells/well in DMEM media (Sigma) containing  
20 penicillin/streptomycin, L-glutamine (GibcoBRL) and incubated overnight at 37 °C. 1 hr before the assay was run, the media was removed and replaced with 100 µl serum free DMEM/well. 100 µl of 5 µM DRAQ5 (Biostatus) in Krebs buffer was added to

each well and incubated for 15 minutes at 37°C. The plate was then placed in the imager (LEADseeker Cell Analysis System) and wells were imaged at varying time points following addition of agonist (50µl of 40 ng ml IL1β). Translocation of the P65-GFP was observed from the cytoplasm to the nucleus following agonist addition.

5 The ratio of nuclear cytoplasmic fluorescence is shown in Figure 7.

The foregoing is illustrative of the present invention and is not to be construed as limiting thereof. Although a few exemplary embodiments of this invention have been described, those skilled in the art will readily appreciate that many modifications  
10 are possible in the exemplary embodiments without materially departing from the novel teachings and advantages of this invention. Accordingly, all such modifications are intended to be included within the scope of this invention as defined in the claims. Therefore, it is to be understood that the foregoing is illustrative of the present invention and is not to be construed as limited to the specific embodiments disclosed,  
15 and that modifications to the disclosed embodiments, as well as other embodiments, are intended to be included within the scope of the appended claims. The invention is defined by the following claims, with equivalents of the claims to be included therein.